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Expression of a novel human myotonin protein kinase (MtPK) cDNA clone which encodes a protein with a thymopoietin-like domain in COS cells

Noboru Sasagawa[a], Hiroyuki Sorimachi[a], Kei Maruyama[b], Kiichi Arahata[c], Shoichi Ishiura[a,*], Koichi Suzuki[a]

[a]*Institute of Molecular and Cellular Biosciences, the University of Tokyo, Tokyo 113, Japan*
[b]*National Institute for Physiological Sciences, Okazaki 444, Japan*
[c]*National Institute of Neuroscience, NCNP, Kodaira, Tokyo 187, Japan*

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Abstract

A full-length cDNA of human myotonin protein kinase (MtPK) was cloned and expressed in COS-1 cells. MtPK is recovered from the cytosolic fraction of the COS extract as a 70 kDa protein, which coincides with the size deduced from the predicted amino acid sequence. The sequence has a significant homology to thymopoietin, a peptide hormone of the thymus. Biochemical characteristics of MtPK expressed in COS-1 cells and its expression in rat tissues are investigated.

Key words: Myotonic dystrophy; Protein kinase; Thymopioetin; cDNA clone; Triplet repeat

1. Introduction

Myotonic Dystrophy (Dystrophia Myotonica, DM) is an autosomal dominant multi-systemic disorder with an estimated minimum incidence of 1 per 8,000 of the population [1]. It is the commonest form of muscular dystrophy affecting adults, charactarized primarily by myotonia and progressive muscle weakness, although the clinical manifestations vary significantly. Increased severity through the generations, anticipation, is also a unique feature of DM. The mutation underlying DM is the expansion of a polymorphic CTG repeat in the 3′-noncoding region of the gene mapping to chromosome 19q13.3 [2–6]. This DM cDNA has been cloned by several laboratories, and has been shown that the sequence has homology to serine-threonine protein kinase (MtPK), although there are minor discrepancies in the nucleotide and deduced amino acid sequences [2,7,16]. The exact function of MtPK protein remains, however, unknown. There are inconsistent reports concerning the length of the CTG repeats and the relationship of mRNA expression to disease severity [8–14]; the number of CTG repeats in the normal population varies between 5 and 37. $(CTG)_{30-37}$ could possess predisposing features making it a reservoir for new DM mutations. Decreased and increased disease expression has been reported as the number of CTG repeats increases.

To investigate the role of CTG repeats in the expression of the MtPK gene, we cloned a full-length cDNA for MtPK and its nucleotide sequence was determined. MtPK expression has been analyzed by several groups, with apparently conflicting results concerning the levels of MtPK mRNA and protein products being obtained [4–6]. Caskey et al. reported reduced mRNA levels in adult tissues using the RT-PCR method [10,12]. According to Korneluk, however, the increased mRNA levels in samples from congenital patients were striking [14]. Antibodies against a fusion-protein of MtPK crossreact with a 55 kDa protein [12,15], a size slightly smaller than the value estimated from cDNA sequence analysis. In order to address these discrepancies, we transiently expressed a full-length cDNA clone in COS-1 cells and examined protein expression by Western blot analysis. The expressed MtPK has a M.W of 70 kDa and the protein was isolated from the cytosolic fraction of transfected COS cells.

2. Materials and methods

2.1. Screening and sequencing of the MtPK cDNA clone

Two overlapping clones corresponding to about 700 bp of the 5′ region and about 1,800 bp of the 3′ region of the MtPK cDNA clone were isolated from an adult human muscle cDNA library (CLONTECH) using synthetic oligonucleotides (CGGCCCGGGGAGGGGCCATGGTG and CCTGCAGGCTGTGGGCGGTGGG for 5′ region [8] as PCR primers and oligos 101 and 102 in [2] for 3′ region as a probe). Each clone was digested with *Sse*8387I and a ligated full-length clone was introduced into pUC118. The sequence of the MtPK full-length cDNA clone is shown in Fig. 1.

2.2. Expression in COS cells and Western blot analysis

The MtPK cDNA obtained above was inserted into a vector pSRD, and transfected into COS-1 cells by the DEAE-dextran method. Electroporation was also used for more effective transfection. After 48 h incubation, transfected cells were harvested and sonicated in 20 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl (buffer A). After centrifugation at 15,000 × *g* for 3 min, the supernatant was used for enzyme assay.

*Corresponding author. Fax: (81) (3) 3813 0654.

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The precipitate was resuspended in buffer A and used for assay. A part of the supernatant was solubilized in an SDS sample buffer (2% SDS, 0.125 M Tris-HCl, pH 6.8, containing 30% glycerol, 5% 2-mercaptoethanol, and 0.02% Bromophenol blue) and boiled for 2 min. Samples were loaded onto 10% SDS polyacrylamide gels for electrophoresis. After electrophoresis, proteins were electroblotted onto nitrocellulose membranes, and the filters were soaked in antibody solution in 25 mM phosphate buffer, pH 7.4, containing 0.5 M NaCl and 0.05% Tween-20. Proteins were visualized with a VECTASTAIN ABC kit (rabbit IgG, Vector Lab).

The antibodies used for analyses were raised in rabbits using peptides synthesized from the predicted peptide sequence as antigens. Antibody DM1 recognizes MtPK amino acid residues 281–296 (DSTAETYGKIVHYKEH), while antibodies DM5 and DM2 recognize the putative (LNPRTVFDSGAPLED) peptide corresponding to amino acid residues 623–637 [7], which is now known to be a frame-shift product according to our sequence (PEP*NCLRLRGPVGR), and (EAEARNRDLEAHVRQ) corresponding to amino acid residues 500–514, respectively (Koga, R. et al., submitted). All peptides were synthesized by a peptide synthesizer (Applied Biosystems, Inc.).

2.3. Enzyme assay

Supernatant fractions of transfected COS-1 cell extracts were immunoprecipitated with anti-DM1 antibody/Protein A-Sepharose. The precipitates were washed in a washing buffer (25 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl, 20% glycerol, and 0.1% Nonidet P-40) three times and resuspended in buffer A. These immunoprecipitated samples were used for protein kinase and ATPase assays.

2.4. In vitro phoshorylation assay of MtPK

c-AMP, Ca, phospholipid and Ca, CaM-dependent protein kinase activities were determined. Phosphorylation of casein and autophosphorylation of MtPK itself were examined. Immunoprecipitated protein samples were mixed with 10 mM 2-mercaptoethanol, 20 mM ATP, 1 mCi [γ-^{32}P]ATP, 5 mM $MgCl_2$, 0.15 M NaCl (total vol.: 40 ml). 1 mM c-AMP was added to determine c-AMP dependent protein kinase, 2 mM Ca^{2+} and 2.5 mM CaM for Ca, CaM-dependent kinase, and 2 mM Ca^{2+}, 4 mg/ml diolein, 50 mg/ml PIP_2, and 50 mg/ml PS for Ca- and phospholipid-dependent kinase assays. Samples were incubated at 30°C for 1 h and loaded onto SDS-PAGE (10% gels). The electrophoresed gels were dried and analyzed by a Bio-Image analyzer (Fuji Film).

2.5. In vivo phosphorylation assay

Proteins in transfected COS-1 cells were labeled directly by [^{32}P]orthophosphate. Confluent cells in a 6 cm-dish were first washed several times with saline, then 1 ml of non-P_i medium containing 5% dialyzed serum (at 0.15 M NaCl) and 100 mCi/dish [^{32}P]orthophosphate were added and the mixtures were incubated for 3 h. After washing, the ^{32}P-labeled cells were soaked in 0.1% Triton X-100 and the solubilized proteins were centrifuged at $15{,}000 \times g$ for 3 min. The resultant supernatant was immunoprecipitated with anti-DM1. Proteins were loaded on SDS-PAGE (10% gel) and electrophoresed gel was analyzed by Bio-Image Analyzer (Fuji Film).

2.6. Assay of ATPase activity

Immunoprecipitated sample was incubated in 10 mM ATP, 50 mM Tris-HCl, pH 7.0, and metal ions. Four kinds of ions were examined; 0.1 M NaCl, 16 mM $CaCl_2$, 0.1 M KCl and 5 mM $MgCl_2$ (final concentration). Each sample was incubated at 37°C for 30 min and reaction was stopped by 10% TCA (trichloroacetic acid). After centrifugation at $500 \times g$ for 15 min, 100 ml of sample was mixed with 200 ml of a Mo-reagent (2.5% ammonium molybdate/5 N H_2SO_4) and 300 ml of 10% L-ascorbic acid. After incubation at 30°C for 10 min, the liberated phosphate was measured at 660 nm (Hitachi spectrophotometer F3000).

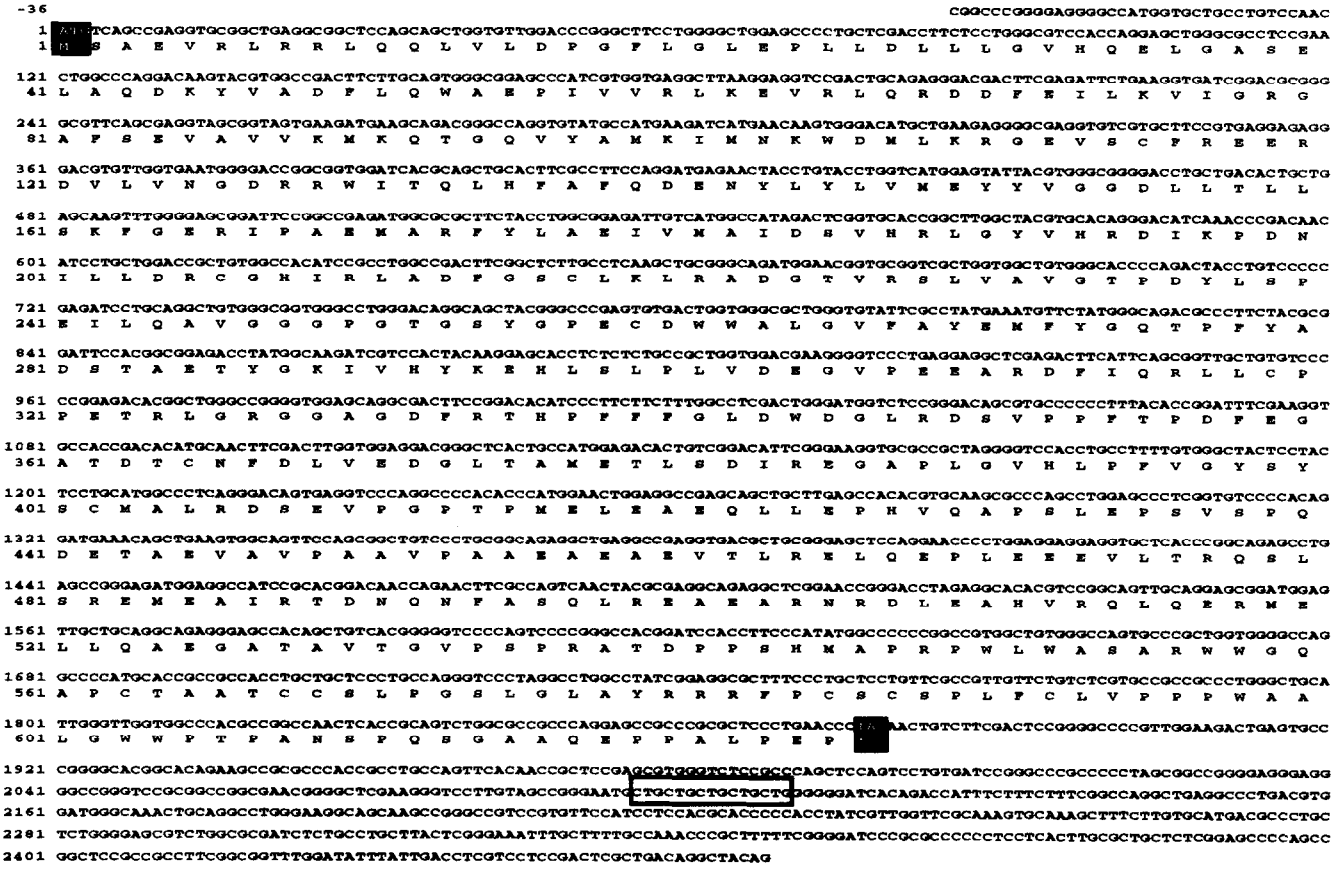

Fig. 1. Nucleotide and amino acid sequences of human MtPK. The complete alignment of human MtPK cDNA is shown, together with the predicted protein sequence. The ATG start codon and TGA termination codon are shown by reversed letters. The box highlights the $5 \times$ CTG repeat.

Fig. 2. Comparison of the N-terminal amino acid sequence deduced from the nucleotide sequence of MtPK with sequences of thymopoietins (TPs). Lines: 1, human MtPK; 2, bovine TP-I; 3, bovine TP-II; 4, bovine splenin; 5, human TP; 6, human splenin. Reversed letters indicate residues where at least one other peptide shows identity. Peptide sequence with T-cell differentiation activity is underlined.

3. Results and discussion

Fig. 1 shows the nucleotide and deduced amino acid sequences of a newly-isolated full-length MtPK cDNA clone. The sequence is essentially identical to that previously described, but differs slightly near the termination codon. The termination codon is at nucleotide 1,876 rather than at 1,869 [2] or 1,913 [7,16]; the putative molecular weight was calculated as 69,617 Da, close to the reported value [8,16].

A computer search of the protein data base indicates that the N-terminal amino acid sequence predicted from the nucleotide sequence of MtPK shows a considerable degree of similarity to many previously sequenced thymopoietins (TPs), a 49-amino acid (48- for human) hormone of the thymus with pleiotropic actions on prothymocytes, mature T-cells and nicotinic receptors [17,18]. Recent report casts doubt about interaction of TP with both the muscle- and neuronal-type nicotinic α-bungarotoxin receptors [19]. These include bovine TPs, splenin and their human counterparts. Fig. 2 shows the sequence alignment of bovine TPs, human TP and splenins with the amino acid sequence predicted from MtPK cDNA clone. The N- terminal domain of MtPK (11–60) shows approximately 32% amino acid identities to the entire region of TPs. These sequence similarities between MtPK and other known TPs may suggest a specific role of MtPK in immune cells. However, because the similarity between MtPK and TPs was not so high, we were unable to use the sequence data to predict the hormonal function of the MtPK coded by a novel cDNA. The isolation of MtPK should contribute to the further understanding of this protein in DM.

This MtPK cDNA clone was inserted into a pSRD vector, and transfected into COS cells. The transfected COS cells produced a 70 kDa protein identical to the estimated value (Fig. 3). This protein localized exclusively to the supernatant fraction. No degradation products were observed in the COS-extracts. In addition, endogenous MtPK was not detectable in COS-1 cells. Anti-DM1 was used as the first antibody in these experiments. Anti-DM5 did not crossreact with the 70 kDa protein. These results suggest that the sequence of peptide DM1 is certainly involved in the expressed protein whereas the frame-shift product, derived from the sequence published in [2], is not a translational product from MtPK mRNA.

The MtPK cDNA clone shows a sequence conserved among Ser/Thr protein kinases. In order to determine whether the 70 kDa protein is a protein kinase, its kinase activity was examined. cAMP-dependent and/or calcium-dependent protein kinase activities were determined using ^{32}P-labeled ATP and casein as substrates. So far we have been unable to detect protein kinase activities. Autophosphorylation of the 70 kDa protein was also measured. After incubation of the COS-1 supernatant with ^{32}P-ATP and Mg^{2+}, the proteins were immunoprecipitated with anti-DM1 and electrophoresed. No increase in the intensity of the band corresponding to molecular weight about 70 kDa was observed. ATP-

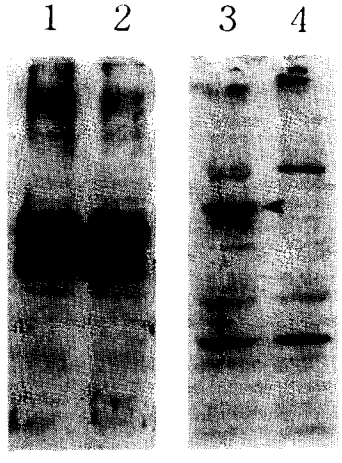

Fig. 3. Immunoblotting of cDNA-transfected COS cell extracts. Freshly prepared COS-1 cells were homogenized with a Dounce homogenizer, and the homogenate was centrifuged at 15,000 × *g* for 10 min. The resultant precipitate (lanes 1 and 2) and supernatant (lanes 3 and 4) were solubilized with 2% SDS and electrophoresed in 10% SDS gels. Fractionated proteins from MtPK cDNA-transfected (lanes 1 and 3) and vector-transfected (lanes 2 and 4) COS-1 cells are shown. Several non-specific bands can be observed in this blot; however, these bands disappeared when antibody against the *E. coli*-generated C-terminus portion of MtPK (from nucleotide 1,246 to the C-terminus, K. Maruyama, unpublished results) was used as a primary antibody. Expressed MtPK is indicated by an arrowhead. M, low-molecular-weight markers, phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and lactoalbumin (14 kDa).

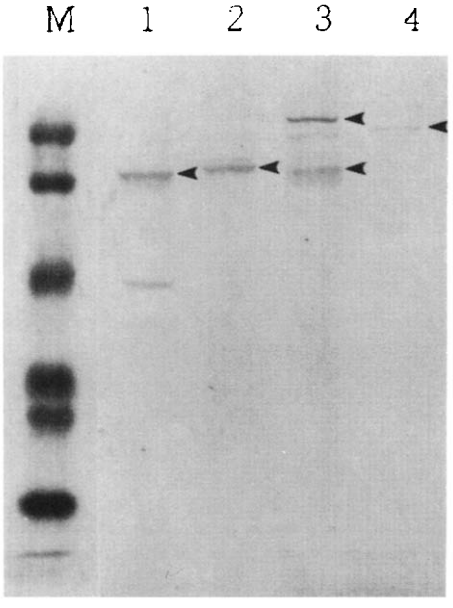

Fig. 4. Expression of MtPK in adult rat tissues. Ten micrograms of protein from rat tissue extracts were applied to each lane and visualized by anti-DM1 antibody after immunoblotting (10% SDS gel). Crossreactive proteins with anti-DM1 and anti-DM2 (data not shown) are designated by arrowheads. Lane M, marker proteins as described in Fig. 2. Lane 1, cardiac muscle; lane 2, brain; lane 3, liver; lane 4, lung.

ase activity was also determined in the presence and absence of Ca^{2+}, Mg^{2+}, K^+ and Na^+, since the nucleotide sequence of MtPK appeared to be similar to that of myosin [2]. Only a 1.2-fold increase in ATPase activity was observed in 0.1 M Na^+ solution. Autophosphorylation of endogenous MtPK was not observed in cells labeled with ^{32}P-orthophosphate and immunoprecipitated with anti-DM1. These results indicate that the 70 kDa MtPK protein is inactive under the assay conditions used. Specific co-factor(s) or MtPK processing might be required for activation.

As shown in Fig. 4, Western blot analysis of cytosolic proteins with anti-DM1 showed a full-length MtPK (70 kDa) and a shorter processed polypeptide (43 kDa) to be present in cardiac muscle. The latter 43 kDa band disappeared when anti-DM2 was used as a primary antibody (unpublished results), indicating that this band was not a protein product of a MtPK gene. Longer polypeptides were also detected in liver and lung extracts. There are several reports indicating that a 54 kDa or 55 kDa protein is recognized by anti-MtPK antibody [10,15]. Therefore the low-molecular weight MtPK protein found in this and previous reports might have arisen through some modifications or degradation of the full-length precursor to an active form.

Korneluk et al. suggested that there are two mRNA isoforms of MtPK, one coding a protein of 69 kDa (skeletal isoform) and the other a protein of 60 kDa (cardiac isoform) [14]. Therefore, alternative splicing of MtPK mRNA might occur. This alternative splicing may also play a role in the function of MtPK. The level of the expressed 70 kDa protein was significantly lower than that usually observed for transfections of other cDNAs by the same method. Therefore, the stability of the expressed MtPK 70 kDa protein must be considered.

Myotonia is clinical feature of DM [1]. Biochemical features include membrane abnormalities, decreased phosphorylation of membrane-bound proteins, changes in the Ca^{2+}-permeability of erythrocyte ghosts, a modulation of ATPase activity, and a decrease in membrane fluidity. It therefore appears that DM is a systemic disease of neuromuscular transmission and/or signal transduction. MtPK may play an important role in these functions [20]. If so, either increased or decreased expression of MtPK in tissue can cause myotonia, an abnormality of neuromuscular transmission.

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